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Antigen-receptor induced clonal expansion and deletion of lymphocytes are impaired in mice lacking HS1 protein, a substrate of the antigen-receptor-coupled tyrosine kinases

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HS1, an intracellular protein expressed specifically in hematopoietic cells, is rapidly tyrosine phosphorylated after cross-linking of antigen receptors on B and T lymphocytes, implicating involvement of this molecule in the signal transduction pathways from the antigen receptors as a substrate of membrane-associated tyrosine kinase(s). The development of lymphoid cells in HS1-deficient mice, generated through gene targeting, appeared normal. However, antibody production to T-independent antigen and proliferative responses of splenic B and T cells after cross-linking of the antigen receptors were impaired in these mutant mice. Furthermore, B cells in the peritoneal cavity of the mutant mice were resistant to multivalent cross-linking of the antigen receptor, which causes apoptosis of such cells in normal mice. Crossing the HS1-deficient mice with the mice harboring transgenes encoding α and β chains of T-cell antigen receptor against a male H-Y antigen resulted in a progeny that demonstrated a significantly impaired ability of thymic negative selection. These results indicate that HS1 is a novel molecule involved in the antigen-receptor-derived signaling pathways and plays important roles not only in clonal expansion, but also in clonal deletion of B and T cells.

Keywords: antigen receptors/clonal deletion/clonal expansion/gene targeting/HS1

Introduction

Membrane-bound antigen receptors, surface immunoglobulin (sIg) on B cells (B-cell antigen receptor, BCR) and T-cell antigen receptors (TCR) on T cells, when cross-linked by an antigen, produce signals leading to differentiation, activation and/or proliferation of the lymphocytes. In the early stages of lymphocyte development, expression of immunoglobulin (Ig) μ chains or TCR β chains, which form antigen-receptor-like complexes on the cell surface, is essential for differentiation of lymphocyte precursors (Kitamura *et al.*, 1991; Mombaerts *et al.*, 1992). In the peripheral lymphoid tissues, recognition of an exogenous antigen through the surface antigen receptors results in activation and proliferation of mature B and T cells, and eventually induces differentiation of B cells into antibody-secreting cells, and of T cells to exert their cytotoxic or helper activity. In addition, antigen receptors may also provide signals leading to lymphocyte death by apoptosis (Smith *et al.*, 1989; Benhamou *et al.*, 1990; Hasbold and Klaus, 1990; Shi *et al.*, 1990), which is thought to be an important mechanism for the elimination of self-reactive lymphocytes (negative selection) and, thus, for the induction of immunological tolerance (Murphy *et al.*, 1990; Swat *et al.*, 1991; Murakami *et al.*, 1992; Hartley *et al.*, 1993). Currently, it is not known how the signals from the antigen receptors are regulated to show such diverse responses of the cells, and it is one of the most important issues for understanding how immunological tolerance is maintained without suppressing the immune response to foreign antigens.

After cross-linking of the BCR, one of the earliest detectable events in the cells is the activation of non-receptor-type protein tyrosine kinases (NR-PTKs), such as Lyn, Blk, Fyn and Syk (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Hutchcroft *et al.*, 1992), followed by rapid tyrosine phosphorylation of a number of intracellular substrates, including phosphatidyl inositol 3-kinase (Yamanashi *et al.*, 1992), phospholipase Cγ1 and Cγ2 (Bijsterbosch *et al.*, 1985), Ras GTPase-activating protein (Gold *et al.*, 1992), Vav (Bustelo and Barbacid, 1992), Shc (Saxton *et al.*, 1994) and other unidentified proteins (Campbell and Cambier, 1990; Gold *et al.*, 1990). These phosphorylated substrates are believed to propagate further signals in cascades that eventually reach to the nucleus, where the transcription of a number of genes including immediately early genes, such as c-*myc*, c-*fos*, *egr-1* or *junB*, is induced (Kelly *et al.*, 1983; Monroe, 1988; Seyfert *et al.*, 1989; Tilzey *et al.*, 1991). The protein products of such genes are transcription factors and may be important intermediates in determining the lymphocyte responses after antigen stimulation. For T cells, cross-linking of the TCR–CD3 complex induces rapid activation of NR-PTKs such as Lck, Fyn and ZAP70, followed by the propagation of signals very similar to those observed in B cells (reviewed by Weiss and Littman, 1994).

Recent studies utilizing genetic approaches have revealed that the activation of NR-PTKs is functionally important in the signaling pathways leading to lymphocyte development, proliferation or both. In mice with null mutation of the *lck* gene, the development of thymocytes was profoundly blocked at the $CD4^-CD8^-$ double-negative (DN) stage, and the leaked T cells in the periphery of these mice exhibited an impaired proliferative response to TCR–CD3 cross-linking (Molina *et al.*, 1992). In mice lacking Fyn in lymphocytes, lymphocyte development was normal, but cross-linking of the TCR–CD3 complex led to impaired proliferation and calcium influx in both thymocytes and splenic T cells (Appleby *et al.*, 1992; Stein *et al.*, 1992). Mutations in *ZAP-70* gene causing loss of kinase activity of ZAP-70 were discovered in selective T-cell deficiency patients, who showed a block of differentiation from $CD4^+CD8^+$ double-positive (DP)

thymocytes into $CD8^+$ single-positive (SP) thymocytes and an impaired proliferative response of peripheral $CD4^+$ T cells to TCR stimulation (Arpaia *et al.*, 1994; Chan *et al.*, 1994; Elder *et al.*, 1994). The *CD45* gene encodes a surface protein-tyrosine phosphatase which presumably upregulates the activities of NR-PTKs through their dephosphorylation (Charbonneau *et al.*, 1988; Shiroo *et al.*, 1992; McFarland *et al.*, 1993). In mice with a mutation in exon 6 of the *CD45* gene, thymocyte development was blocked at the transitional stage from DP to SP cells, although the development of B lymphocytes was normal. The proliferative responses of peripheral T and B cells from these mice to the antigen-receptor cross-linking were impaired (Kishihara *et al.*, 1993). Finally, *btk*, a member of a new NR-PTK gene family distinct from the *src* family or others, was identified as the responsible gene for X-linked agammaglobulinemia (XLA) in humans (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993) and X-linked immunodeficiency (*xid*) in mice (Rawlings *et al.*, 1993; Thomas *et al.*, 1993). In XLA patients, B-cell development is arrested at the pre-B cell stage, and in *xid* mice, B-cell proliferative response after sIgM stimulation is impaired, and the immune response to type II T-independent antigen is abolished (Scher, 1982; Rigley *et al.*, 1989). NR-PTKs are also implicated in mediating the signals from the antigen receptors leading to growth arrest or apoptosis of lymphocytes. Experiments using antisense oligonucleotides to downregulate the gene expression suggested that growth arrest of a certain B-lymphoma induced by cross-linking of sIgM requires Lyn (Scheuermann *et al.*, 1994), and apoptosis of another B-lymphoma requires Blk (Yao and Scott, 1993).

As suggested by the above observations, different NR-PTKs seem to have distinct as well as overlapping functions in the signaling pathways from the antigen receptors. To clarify the molecular mechanisms of how these NR-PTKs transmit diverse and partly redundant signals to the downstream, a direct approach would be to identify molecules residing downstream of NR-PTKs and to elucidate the functions of such molecules. Several intracellular substrates of the NR-PTKs have been identified, as mentioned above, none of which has proved, so far, to be functionally involved in the induction of the lymphocyte responses, such as differentiation, proliferation, growth arrest or apoptosis, by the antigen-receptor cross-linking.

The *HS1* gene, whose expression is limited to hematopoietic cells, encodes an intracellular protein, HS1, having a mol. wt of ~75 kDa (Kitamura *et al.*, 1989). The amino-terminal half of the HS1 protein contains a similar sequence to the consensus for the basic DNA-binding motifs of the Jun-, Fos-, Myc- and other helix–loop–helix protein families (Prendergast and Ziff, 1989). It also contains three and one-half times repeat of a 37-residue unique motif, each of which contains a helix–turn–helix structure similar to those in several prokaryotic transcription factors (Laughon and Scott, 1984). At the carboxyl-terminal half, HS1 possesses the acidic and amphipathic α-helix structure that is seen in activation domains of transcription factors (Ptashne, 1988). In addition, a Src-homology (SH) 3 motif (Koch *et al.*, 1991) is present near the carboxyl terminus. Thus, HS1 contains several unique motifs that are conserved in transcription factors or signal transduction molecules. The HS1 is highly conserved between human and mouse, especially in the amino-terminal half and the SH3 region (Kitamura *et al.*, 1995). Recently, the HS1 protein was shown to be associated with Lyn kinase and tyrosine phosphorylated rapidly after cross-linking of sIgM on B-lymphoma cells (Yamanashi *et al.*, 1993). It was also shown that the tyrosine-phosphorylated HS1 protein bound to SH2 domains of *src* family NR-PTKs such as Lyn, Fyn and Blk with the highest affinity among the tyrosine-phosphorylated proteins from B-lymphoma cells stimulated with anti-IgM antibody (Baumann *et al.*, 1994). These data suggest that HS1 may be involved in the signal transduction pathways from sIgM as a substrate of NR-PTK(s).

To investigate the functions of the HS1 protein *in vivo*, we have generated HS1-deficient mice through the germline transmission of the *HS1* gene alleles which have been disrupted in mouse embryonic stem (ES) cells by the gene targeting technique (Capecchi, 1989). From analysis of the mutant mice, we demonstrate here that proliferative responses to cross-linking of antigen receptors are impaired in both splenic B and T cells from the HS1-deficient mice, and that B cells in the peritoneal cavity of the mutant mice are resistant to multivalent cross-linking of sIgM, which causes apoptosis of such cells in normal mice. In addition, as evidenced by crossing the HS1-deficient mice with anti-self TCR-transgenic mice, negative selection of thymocytes is partially impaired in the HS1-deficient mice. These results indicate that HS1 plays an important role in the signal transduction pathways initiated at the antigen receptors on both B and T lymphocytes and leading to their proliferation or death.

Results

Generation of HS1-deficient mice

For the construction of the *HS1* gene targeting vectors, we screened a Balb/c genomic DNA library with the mouse *HS1* cDNA and isolated four independent phage clones which together spanned >23 kb of the mouse *HS1* gene locus (Kitamura *et al.*, 1995). Using the genomic fragments from two of these phage clones, two kinds of the targeting vectors, pHS1 5′ko and pHS1 3′ko, were constructed (Figure 1A and B). The pHS1 5′ko vector contained a 5.1 kb genomic fragment of the mouse *HS1* gene in which the 0.3 kb *Hin*cII–*Hin*dIII fragment including the second exon was replaced by the neomycin resistance gene (*neo*^{r}; Thomas and Capecchi, 1987) for positive selection. The translational initiation codon (ATG) was present in this exon. The vector contained the herpes simplex virus thymidine kinase gene (HSV-TK) for negative selection against non-homologous recombinants (Mansour *et al.*, 1988). In the pHS1 3′ko vector, the tenth exon of the *HS1* gene was interrupted by an insertion of the *Escherichia coli lac-Z* gene, which aimed to monitor the expression of the targeted *HS1* gene locus in the mouse, and the *neo*^{r} lacking a poly(A) signal. The targeted locus with this vector could potentially produce a truncated HS1 protein comprised of Met^1–Gly^{237} residues, which would not contain the acidic and amphipathic α-helix structure and the SH3 region.

ES cells (E14; Hooper *et al.*, 1987) were electroporated with either of the two targeting vectors, and selected with both G418 and gancyclovir (GANC) for the pHS1 5′ko,

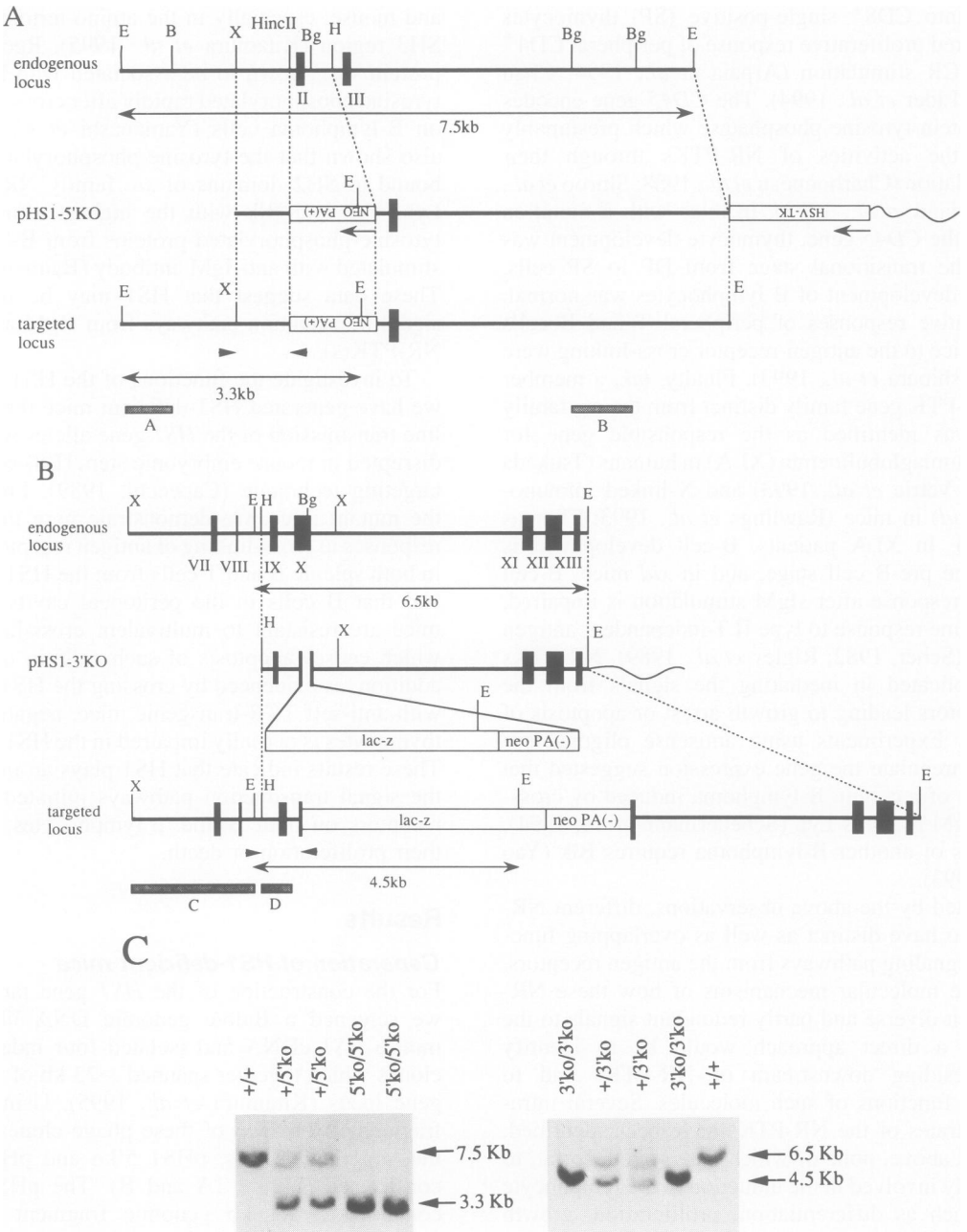

Fig. 1. Homologous recombination at the *HS1* gene locus. (**A**) Structure of the murine *HS1* gene locus, pHS1 5′ko vector and the targeted locus. Introns are indicated by horizontal lines, exons (II and III) by closed boxes, *neo*^{r} and HSV-TK cassettes by open boxes, plasmid vector sequence by a wavy line. Transcriptional orientation of *neo*^{r} and HSV-TK genes is indicated by arrows. PCR primers and the DNA probes (A and B) used to detect homologous recombination events are indicated by arrowheads and stippled boxes, respectively. Genomic DNA fragments from the wild-type and the targeted loci generated by *Eco*RI digestion are shown by double-headed arrows. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; PA, polyadenylation signal; *neo*^{r}, neomycin-resistance gene: HSV-TK, herpes simplex virus thymidine kinase gene. (**B**) Structure of the murine *HS1* gene locus, pHS1 3′ko vector and the targeted locus. Symbols are the same as above, except that lac-Z cassettes are represented by an open box. Exons (VII–XIII) and DNA probes (C and D) are also shown as in (A). (**C**) Southern blot analyses of 4-week-old offspring from matings of *HS1*$^{5'ko}$ or *HS1*$^{3'ko}$ heterozygotes. Genomic DNA from mouse tail was digested with *Eco*RI and hybridized with probe A for *HS1*$^{5'ko}$ mating (left panel) or probe D for *HS1*$^{3'ko}$ mating (right panel). Probe A detected the 7.5 kb wild-type or 3.3 kb mutant band, and probe D detected the 6.5 kb wild-type or 4.5 kb mutant band.

and G418 alone for the pHS1 3′ko. The drug-resistant colonies were screened by the polymerase chain reaction (PCR) for the homologous recombinantion events, which were then confirmed by Southern blot analysis using at least two different probes (Figure 1A and B, data not shown). The *HS1* gene-disrupted ES-cell clones were injected into C57BL/6 blastocysts to generate chimeric mice. The chimeric mice were crossed to C57BL/6 females and the resultant agouti offspring heterozygous for the mutation were intercrossed, then finally the mice homozygous for the targeted loci (*HS1*$^{5'ko}$ or *HS1*$^{3'ko}$) were obtained (Figure 1C). Mating of the heterozygotes yielded wild-type (*HS1*$^{+/+}$), heterozygous (*HS1*$^{5'ko/+}$ or *HS1*$^{3'ko/+}$) and homozygous (*HS1*$^{5'ko/5'ko}$ or *HS1*$^{3'ko/3'ko}$) offspring at

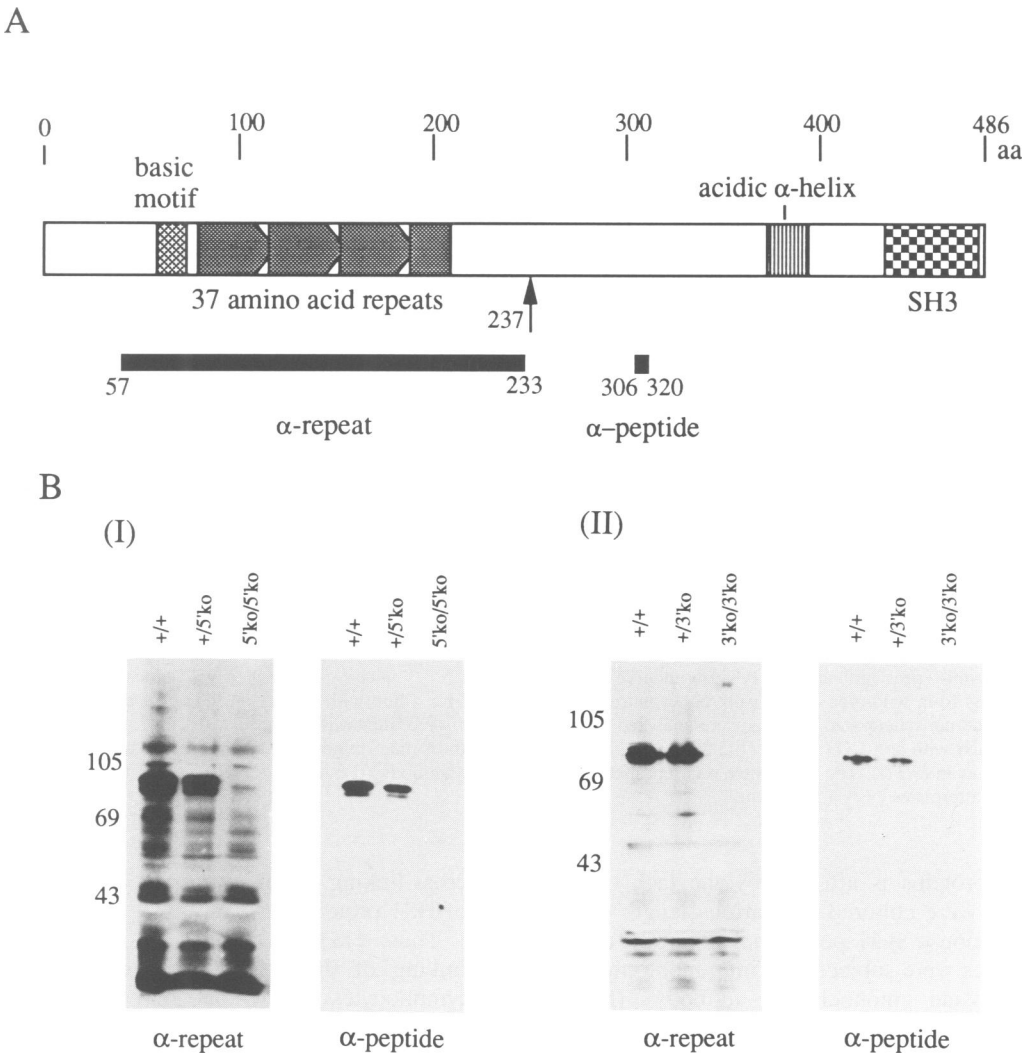

Fig. 2. Absence of the HS1 protein in homozygous $HS1^{5'ko}$ and $HS1^{3'ko}$ mice. (**A**) The top bar represents the predicted structure of the mouse HS1 protein. The arrow indicates the predicted point where translation of the HS1 protein stops in the $HS1^{3'ko}$ allele. The black box (57–233) indicates the region used for constructing the GST–HS1 fusion protein with which a rabbit was immunized to raise anti-repeat antibody. Another black box (306–320) indicates the region corresponding to the synthetic peptide with which a rat was immunized to raise anti-peptide antibody. Abbreviations: aa, amino acids; SH3, Src homology 3. (**B**) Immunoblot analyses with antibodies against HS1 protein. Cell lysates were prepared from spleens of mice with the genotypes indicated on top. HS1 protein was detected with anti-repeat or anti-peptide antibody as indicated.

roughly the expected Mendelian ratio. The animals were housed under conventional breeding conditions. Heterozygous and homozygous mice were fertile, and showed no apparent developmental abnormalities.

The expression of the HS1 protein from the targeted *HS1* loci in splenocytes was analyzed by immunoblot assay with two kinds of antisera against the HS1 protein: one is directed to the repeating motifs (anti-repeat) and the other to a sequence downstream of them (anti-peptide) (Figure 2A). HS1 protein was readily detectable in the lysates of splenocytes from $HS1^{+/+}$ and $HS1^{5'ko/+}$ mice with either of the two antisera, but not at all in those from $HS1^{5'ko/5'ko}$ mice (Figure 2B, I). $HS1^{3'ko/3'ko}$ splenocytes also lacked HS1 protein. The truncated HS1 protein mentioned above could not be detected even by the anti-repeat antiserum which would bind to the truncated protein if present (Figure 2B, II). Moreover, neither β-galactosidase protein nor its enzymatic activity was detected in the splenocytes of $HS1^{3'ko/+}$ and $HS1^{3'ko/3'ko}$ mice (data not shown). Thus, the $HS1^{3'ko}$ allele may be transcriptionally silent or its products may be extremely unstable. In both mutant strains, the expression level of HS1 protein from the heterozygous mice was about half of that from the wild-type mice, indicating that expression of HS1 is gene dosage dependent.

The following results are from the analyses of $HS1^{3'ko/3'ko}$ mice (which are referred to as $HS1^{-/-}$ hereafter), but the same analyses were also performed with $HS1^{5'ko/5'ko}$ mice, except for the experiment crossing them with the TCR-transgenic mouse, and the results were essentially identical for both mutant strains.

HS1 protein is tyrosine phosphorylated in normal B and T cells following cross-linking of antigen receptors

We previously showed that HS1 is one of major tyrosine-phosphorylated proteins in a B-cell lymphoma immediately after cross-linking of sIgM (Yamanashi *et al.*, 1993). First we asked whether HS1 is also tyrosine phosphorylated in normal lymphocytes and whether tyrosine phosphoryl-

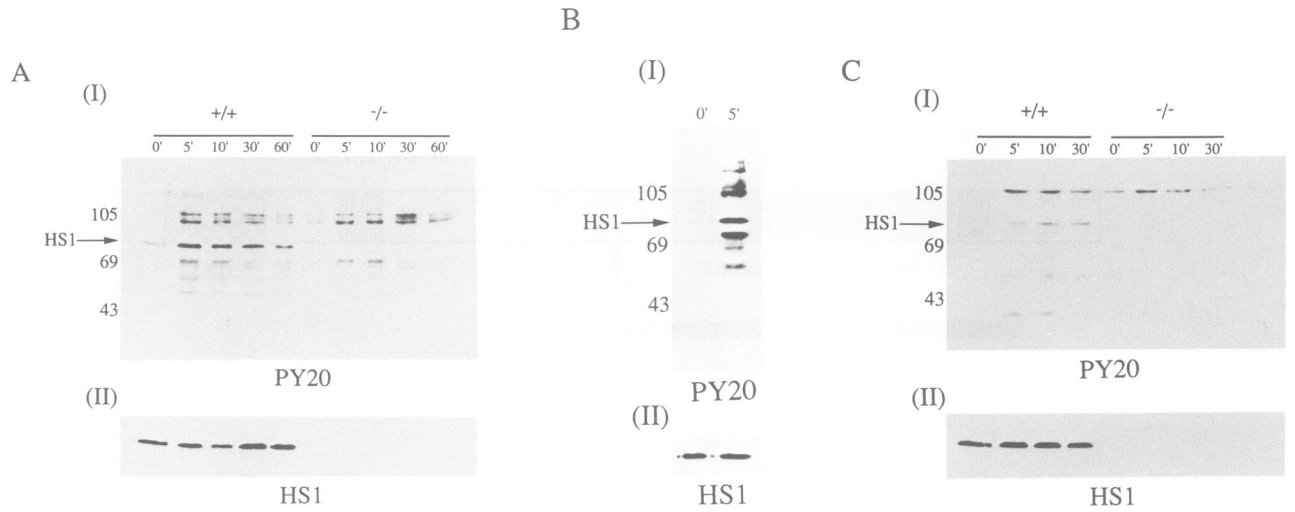

Fig. 3. Protein tyrosine phosphorylation of HS1-deficient lymphocytes following cross-linking of antigen receptors. (**A**) Splenic B cells were incubated for the indicated length of time (minutes) with affinity-purified goat anti-mouse IgM polyclonal antibody. Total cell lysates were prepared and loaded onto an 8% polyacrylamide gel. After transfer to nitrocellulose filter, tyrosine-phosphorylated proteins were detected with HRP-labeled anti-phosphotyrosine monoclonal antibody, PY20 (I). The arrow indicates the location of HS1 protein. The HS1 protein was detected by reprobing the same filter with anti-repeat antibody (II). A representative result using $HS1^{3'ko/3'ko}$ mice (–/–) and their wild-type littermates (+/+) is shown. (**B**) Peritoneal B cells from wild-type mouse were either untreated or incubated for 5 min with affinity-purified goat anti-mouse IgM polyclonal antibody, and tyrosine-phosphorylated proteins were analyzed as in (A) (I). The same filter was reprobed with anti-repeat antibody (II). (**C**) Splenic T cells were incubated with anti-CD3 MoAb (145.2C11) for the indicated length of time (minutes) and tyrosine-phosphorylated proteins in these cells were analyzed as in (A) (I). The same filter was reprobed with anti-repeat antibody (II). A representative result using $HS1^{3'ko/3'ko}$ mice (–/–) and their wild-type littermates (+/+) is shown.

ation of other proteins is affected by the lack of HS1. Splenic B cells were cultured for various length of time with goat anti-mouse IgM polyclonal antibody and the whole-cell lysates were subjected to immunoblotting with anti-phosphotyrosine monoclonal antibody (MoAb), PY20. As shown in Figure 3A, a 75 kDa tyrosine-phosphorylated protein was detected and it reached its maximum 5 min after sIgM cross-linking in wild-type B cells, but was absent in the cells from $HS1^{-/-}$ mice. This 75 kDa protein was identified as HS1 by reprobing the same filter with the anti-HS1 antibody (Figure 3A, II). Tyrosine phosphorylation of the other proteins was also augmented by the cross-linking, but its pattern was very similar between $HS1^{+/+}$ and $HS1^{-/-}$ mice. Thus, tyrosine phosphorylation of HS1 protein is induced in normal splenic B cells by cross-linking of sIgM and the absence of HS1 protein does not apparently affect the tyrosine phosphorylation of other proteins in the B cells. Tyrosine phosphorylation of HS1 upon cross-linking of sIgM was also observed in the peritoneal B cells (Figure 3B).

Protein tyrosine phosphorylation was induced in T-cell lines and splenic T cells following cross-linking of the TCR–CD3 complex by anti-CD3 antibody (June *et al.*, 1990; Stein *et al.*, 1992). We tested whether the HS1 is tyrosine phosphorylated in T cells by the same treatment (Figure 3C). Following stimulation with an anti-CD3ε MoAb, a 75 kDa tyrosine-phosphorylated protein was detected in the lysates of splenic T cells from $HS1^{+/+}$ mice, but not in those from $HS1^{-/-}$ mice, which was identified as HS1 by the reprobing with anti-HS1 antibody. Thus, HS1 is also tyrosine phosphorylated in splenic T cells following the cross-linking of TCR–CD3 complex. Again the number of other tyrosine-phosphorylated proteins and their kinetics of phosphorylation after the cross-linking of CD3 were not grossly altered by the lack of HS1 protein.

Thus, HS1 is tyrosine phosphorylated upon cross-linking of the antigen receptors on normal B and T lymphocytes *ex vivo*, and may be involved in the signal transduction pathways through the antigen receptors as a substrate of protein tyrosine kinase(s) *in vivo*.

Development of lymphoid system is normal in HS1-deficient mice

It has been postulated that the Ig μ chain or TCR β chain form antigen-receptor-like complexes with invariable molecules on the membrane of precursor B cells or DN thymocytes, respectively (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990; Kishi *et al.*, 1991), and the signals from those receptor complexes may play a crucial role in the development of such precursors into more mature stages (Kitamura *et al.*, 1991; Mombaerts *et al.*, 1992). Also, mutations in genes encoding for NR-PTKs, that are supposed to be associated with those receptor-complexes, such as Lck or Btk, were reported to impair the lymphocyte development in mice or humans (Molina *et al.*, 1992; Rawlings *et al.*, 1993; Thomas *et al.*, 1993; Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). Since HS1 was shown to express in cell lines which represent the precursor B or T cells, it might be a substrate of such NR-PTKs and be involved in the early development of lymphocytes. To verify this possibility, HS1-deficient or control mice were sacrificed at 4–8 weeks of age and cells of lymphoid organs were stained with antibodies against several lymphoid-cell surface markers and analyzed by flow cytometry. Spleens and thymi from $HS1^{+/+}$ or $HS1^{-/-}$ mice were of comparable size and cell numbers. The proportion of subsets defined by the expression of B220,

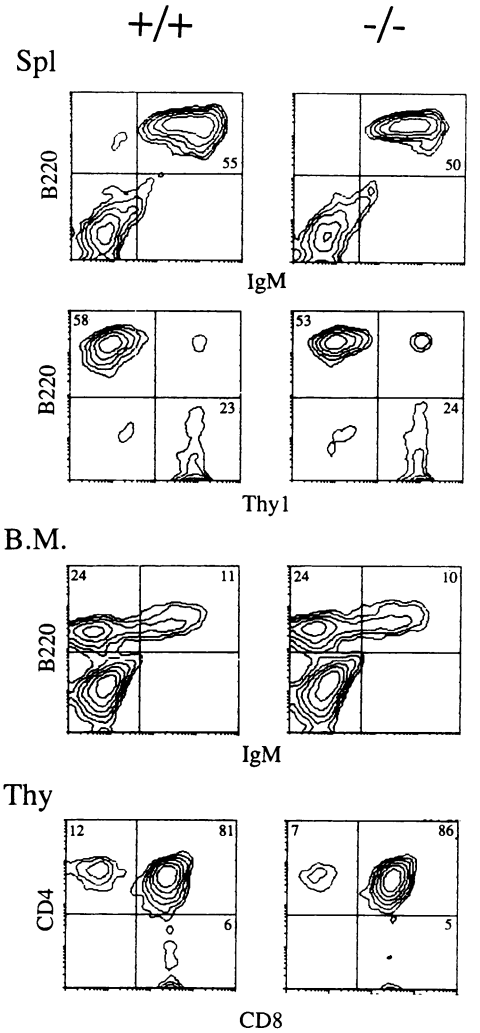

Fig. 4. Normal development of lymphoid cells in HS1-deficient mice. Splenocytes (Spl), bone marrow cells (B.M.) and thymocytes (Thy) from 4- to 8-week-old $HS1^{3'ko/3'ko}$ (–/–) mice or their wild-type littermates (+/+) were stained with the following antibodies: phycoerythin-conjugated (PE–) anti-B220 and fluorescein-conjugated (FITC–) anti-IgM or FITC–anti-Thy1 for splenocytes, PE–anti-B220 and FITC–anti-IgM for bone marrow cells, and FITC–anti-CD8 and PE–anti-CD4 for thymocytes. The stained cells were analyzed by flow cytometry. The results are shown as contour profiles. Numbers in quadrants indicate the percentage of the total lymphocyte population. Shown is a representative result of repeated analyses.

IgM, Thy1, CD4 or CD8 on splenocytes, thymocytes and bone marrow cells of $HS1^{-/-}$ mice did not differ significantly from that of $HS1^{+/+}$ mice (Figure 4). The number and cellularity of lymphocytes in lymph nodes and peritoneal cavity were also unchanged in $HS1^{-/-}$ mice (data not shown). These results indicate that the HS1 protein is not essential for the development of the lymphoid system.

Proliferative response of lymphocytes to antigen-receptor cross-linking is impaired in HS1-deficient mice

The activity of some NR-PTKs was shown to be essential for the proliferative response of lymphocytes after cross-linking of antigen receptors (Appleby *et al.*, 1992; Molina *et al.*, 1992; Stein *et al.*, 1992; Kishihara *et al.*, 1993). Since HS1 is one of the major substrates of NR-PTK(s) in such cells, it may play a crucial role in sIg-mediated proliferation signals. To verify this possibility, we measured the proliferation of splenic B cells from HS1-deficient mice after the cross-linking of sIgM. Small dense splenic B cells from $HS1^{+/+}$ or $HS1^{-/-}$ mice were incubated for 48 h with either lipopolysaccharide (LPS), soluble CD40L or polyclonal goat anti-mouse IgM antibodies, and the incorporation of [^{3}H]thymidine was measured (a representative result is shown in Figure 5A). The proliferation induced by the anti-IgM antibodies, of either the whole molecule or $F(ab')_2$ fragment, was markedly impaired in B cells from $HS1^{-/-}$ mice [29.0 ± 10.6% (n = 4, whole Ig) or 30.6 ± 12.6% (n = 4, $F(ab')_2$ fragment) of the incorporation by the wild-type cells]. The responses of the cells to LPS or soluble CD40L were unaffected by the lack of HS1, in accordance with the absence of tyrosine phosphorylation of HS1 by these stimuli in splenic B cells (data not shown). Impaired proliferation of $HS1^{-/-}$ B cells was also evident when the cells were stimulated by various concentrations of a MoAb against mouse μ chain (Figure 5B). These data indicate that HS1 plays a pivotal role in the induction of proliferation of B cells by the sIgM cross-linking.

As tyrosine phosphorylation of HS1 was observed in splenic T cells after stimulation with anti-CD3ε antibody (Figure 3C), HS1 may also be involved in proliferation signals from TCR–CD3 complex. To test this possibility, T cells purified from spleens of $HS1^{+/+}$ or $HS1^{-/-}$ mice were stimulated with various amounts of anti-CD3ε MoAb immobilized on a plastic plate for 48 h and the cell proliferation was measured by the [^{3}H]thymidine incorporation assay. The result showed that the proliferation induced by the anti-CD3ε MoAb was also reduced, albeit less dramatically, in T cells from $HS1^{-/-}$ mice [a representative result is shown in Figure 5C, mean incorporation of $HS1^{-/-}$ cells at 1 μg/ml anti-CD3ε Ab was 69.6 ± 14.8% (n = 4) of that of the wild-type cells]. Thus, HS1 is necessary for the full proliferative response of T cells mediated by TCR cross-linking. The production of interleukin (IL)-2 and expression level of IL-2 receptor after TCR cross-linking were similar between $HS1^{+/+}$ and $HS1^{-/-}$ mice, and proliferative responses of splenic T cells to IL-2 before and after TCR cross-linking were equivalent between the two (data not shown). Therefore, the impaired TCR-mediated T-cell proliferation in $HS1^{-/-}$ mice does not seem to be due to the deficiency in the induction of IL-2, its receptor or signaling competence of the receptor.

Antibody production of HS1-deficient mice

The humoral immune response of the $HS1^{-/-}$ mice was evaluated by immunizing the mice with T-dependent antigen, NP-chicken gammaglobulin (CG) or type II T-independent antigen, TNP-Ficoll. There was no difference between controls and $HS1^{-/-}$ mice in the serum antibody titer against CG after the former immunization. However, the TNP-specific serum antibody titer after the latter immunization was significantly reduced in $HS1^{-/-}$ mice as compared with those in the control mice (Figure 6). Thus, the ability of B cells in $HS1^{-/-}$ mice to produce a specific antibody to type II T-independent antigens appears to be partially impaired.

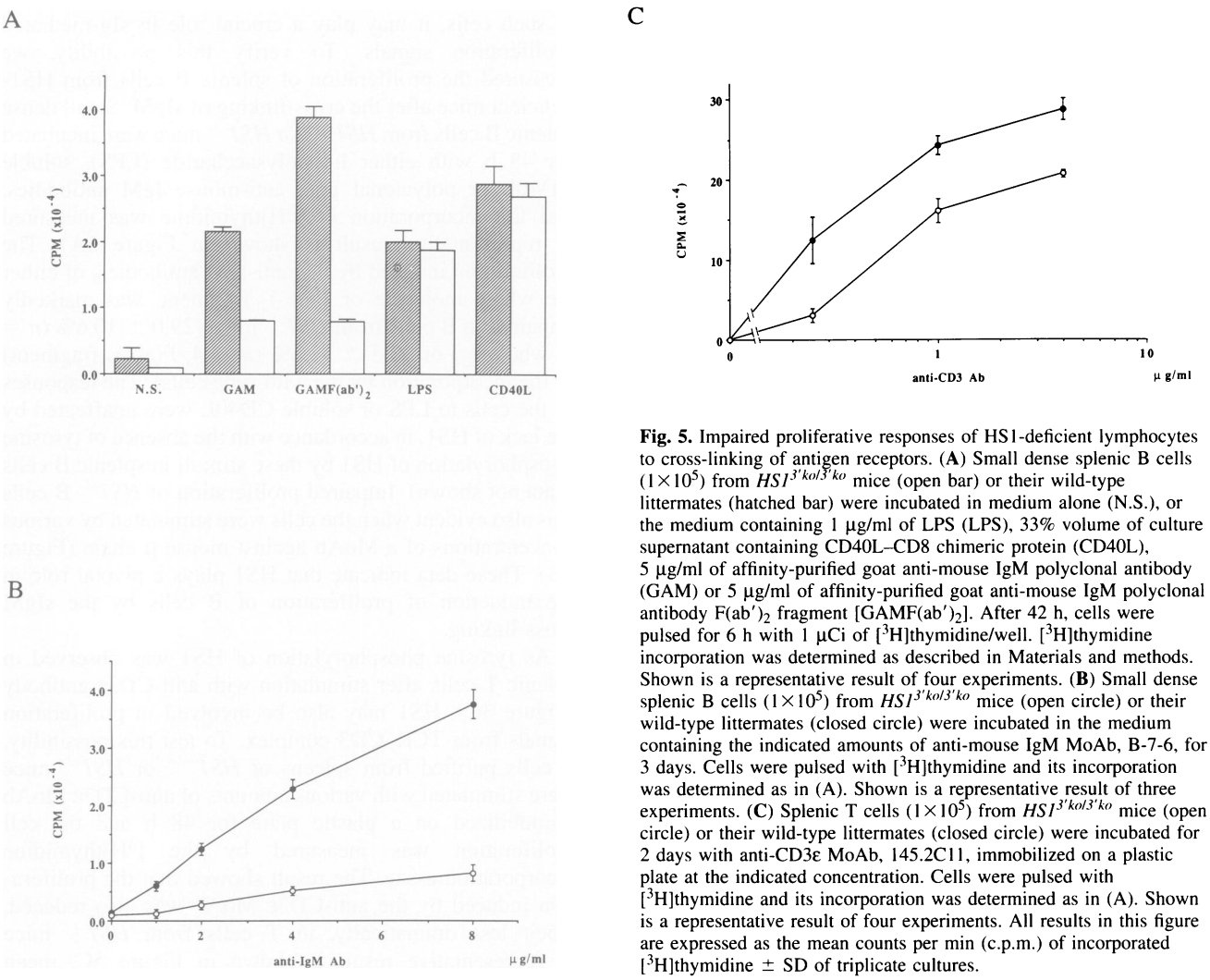

Fig. 5. Impaired proliferative responses of HS1-deficient lymphocytes to cross-linking of antigen receptors. (**A**) Small dense splenic B cells (1×10^5) from $HS1^{3'ko/3'ko}$ mice (open bar) or their wild-type littermates (hatched bar) were incubated in medium alone (N.S.), or the medium containing 1 μg/ml of LPS (LPS), 33% volume of culture supernatant containing CD40L–CD8 chimeric protein (CD40L), 5 μg/ml of affinity-purified goat anti-mouse IgM polyclonal antibody (GAM) or 5 μg/ml of affinity-purified goat anti-mouse IgM polyclonal antibody $F(ab')_2$ fragment [GAMF(ab')$_2$]. After 42 h, cells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine/well. [^{3}H]thymidine incorporation was determined as described in Materials and methods. Shown is a representative result of four experiments. (**B**) Small dense splenic B cells (1×10^5) from $HS1^{3'ko/3'ko}$ mice (open circle) or their wild-type littermates (closed circle) were incubated in the medium containing the indicated amounts of anti-mouse IgM MoAb, B-7-6, for 3 days. Cells were pulsed with [^{3}H]thymidine and its incorporation was determined as in (A). Shown is a representative result of three experiments. (**C**) Splenic T cells (1×10^5) from $HS1^{3'ko/3'ko}$ mice (open circle) or their wild-type littermates (closed circle) were incubated for 2 days with anti-CD3ε MoAb, 145.2C11, immobilized on a plastic plate at the indicated concentration. Cells were pulsed with [^{3}H]thymidine and its incorporation was determined as in (A). Shown is a representative result of four experiments. All results in this figure are expressed as the mean counts per min (c.p.m.) of incorporated [^{3}H]thymidine ± SD of triplicate cultures.

Peritoneal B cells of HS1-deficient mice are resistant to multivalent cross-linking of sIgM

In addition to the proliferative response, signals from antigen receptors lead to apoptosis of some B and T cells (Smith *et al.*, 1989; Benhamou *et al.*, 1990; Hasbold and Klaus, 1990; Shi *et al.*, 1990). Recently, it was shown that the peritoneal B cells in normal mice underwent apoptosis after i.p. administration of both the first anti-IgM antibody and the second antibody against the first one, which would cause strong cross-linking of sIgM (Tsubata *et al.*, 1994). The clonal deletion of the peritoneal B cells was also shown to be induced by cross-linking of sIgM by physiological antigen: peritoneal B cells expressing transgenic sIgM specific for erythrocyte membrane antigen died by apoptosis after injection of erythrocytes (Murakami *et al.*, 1992). These systems allowed us to study *in vivo* a mechanism of signaling from sIgM that causes clonal deletion of self-reactive mature B cells, which is one possible strategy of peripheral B-cell tolerance. To clarify the importance of HS1 in signals leading to the clonal deletion, we tested the *in vivo* effect of the cross-linking of sIgM on the peritoneal B cells in the $HS1^{-/-}$ mice. Twenty-four hours after injection of both anti-mouse IgM MoAb (AK9, rat IgG, the first antibody) and anti-rat κ MoAb (MAR18.5, the second antibody) into the peritoneal cavity of either $HS1^{+/+}$ or $HS1^{-/-}$ mice, peritoneal cells were collected and stained with MoAbs against B220 and IgM or CD5 (Ly-1), and analyzed by flow cytometry. In $HS1^{+/+}$ mice, the proportion of B220$^+$, IgM$^+$ B lymphocytes, both B-1 (Ly-1 B) cell (Ly-1$^+$, B220^{dull}) and B-2 (conventional B) cell (Ly-1$^-$, B220^{high}) subsets, decreased markedly (Figure 7A and B), as reported previously (Tsubata *et al.*, 1994). The decrease in the proportion of B cells was >4-fold on average in repeated experiments (legend to Figure 7). When only the second antibody was injected or anti-HS1 MoAb (rat IgG) was used for the first antibody as a control, the B-cell population was not affected (data not shown). In contrast to the control mice, the peritoneal B cells in the $HS1^{-/-}$ mice were apparently resistant to the same administration of both the first and the second antibodies. The proportion of both the B-1 and the B-2 cell subsets was only slightly reduced in the $HS1^{-/-}$ mice after the treatment (Figure 7, right panels). The small reduction in the proportion of B-cell subsets may be due to the contamination of expanded non-B, non-T inflammatory cells induced by the treatment into the lymphocyte population in the analysis (Tsubata *et al.*, 1994). Alternatively, this may indicate a partial compensation of signals leading to the apoptosis of the B cells. In any case, the results described here indicate that

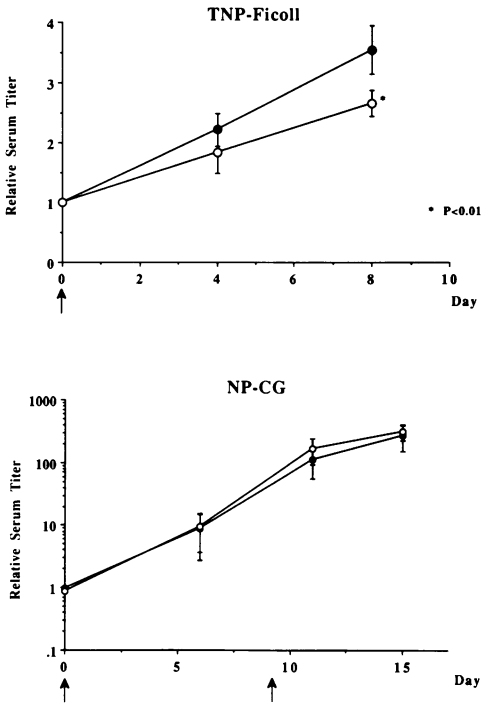

Fig. 6. Humoral immune response to T-independent or T-dependent antigens. Eight-week-old *HS1*$^{3'ko/3'ko}$ mice (open circle, n = 3) and their wild-type or heterozygous littermates (closed circle, n = 4) were challenged with TNP-Ficoll (type II T-independent antigen, **top panel**) or NP-CG (T-dependent antigen, **bottom panel**) on the days shown by arrows. At the indicated day points, mice were bled and the TNP-specific IgM titer (top) or CG-specific IgG titer (bottom) were determined by ELISA as described in Materials and methods. Shown are the means ± SD of antibody titers of serum samples relative to that of a pre-immune serum sample (day 0) from one wild-type mouse.

HS1 is at least one of the molecules playing a functional role in the signaling pathways from sIgM leading to apoptosis of the peritoneal B cells.

Absence of HS1 protein results in impaired negative selection of thymocytes in anti-H-Y antigen αβ TCR transgenic mice

The facts that tyrosine phosphorylation of HS1 was induced in splenic T cells by cross-linking of TCR–CD3 complex, and that the proliferative response of splenic T cells lacking HS1 to the same treatment was impaired, suggest that HS1 is involved in a signaling pathway from the TCR–CD3 complex on thymocytes as well, and participates in positive or negative selection of such cells. To test this possibility, *HS1*$^{-/-}$ mice were crossed to a transgenic (Tg) mice, which carried the α- and β-chain transgenes encoding the TCR specific for H-Y male antigen peptide presented on the H-2D^b molecule (Kisielow *et al.*, 1988). In the male Tg mice of H-2D^b background, DP and CD8$^+$ SP thymocytes are eliminated, possibly through apoptosis of the DP cells at a very early stage, which is considered as an ideal *in vivo* model of thymic negative selection (Kisielow *et al.*, 1988; Swat *et al.*, 1991). HS1-deficient and control mice analyzed here possessed the mixed genotypic background of (129/Ola×C57BL/6), thus they all expressed H-2D^b molecules. As previously reported, due to the negative selection, the number of total thymocytes in *HS1*$^{+/+}$/H-Y Tg male mice decreased

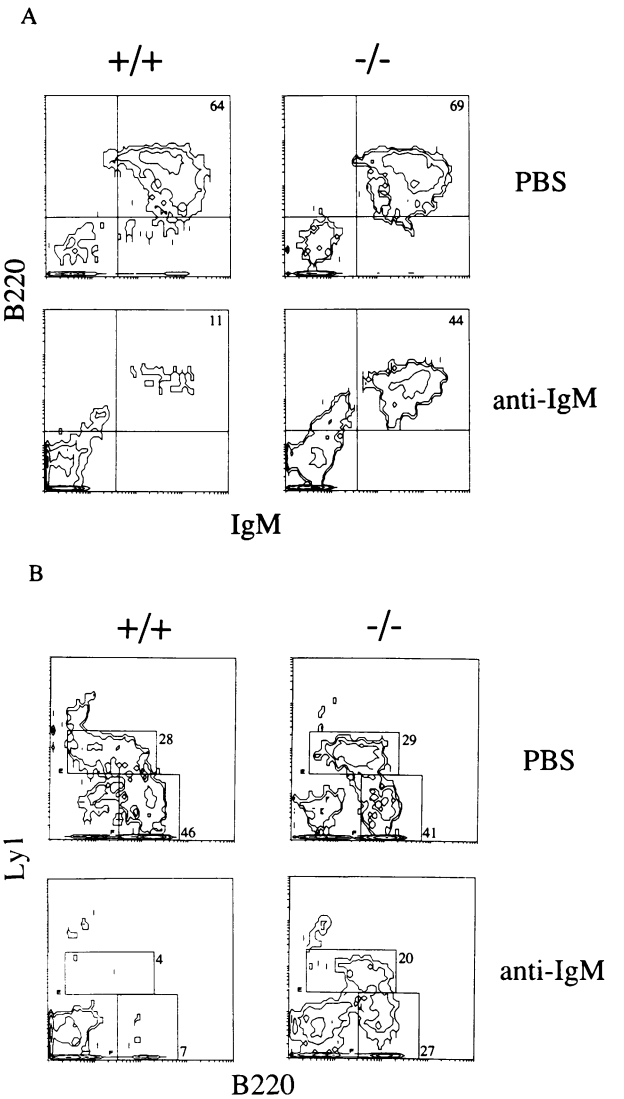

Fig. 7. Peritoneal B cells from HS1-deficient mice are resistant to multivalent cross-linking of sIgM. A total of 150 μg each of MoAbs, anti-IgM (AK9, rat IgG) and, 30 min later, anti-rat κ (MAR18.5) were injected into the peritoneal cavities of 4- to 6-week-old *HS1*$^{3'ko/3'ko}$ mice (–/–) or their wild-type littermates (+/+). PBS was injected as a control. After 24 h, peritoneal cells were collected and stained with PE–anti-B220 and FITC–anti-IgM (**A**) or PE–anti-Ly1 and FITC–anti-B220 (**B**). Stained cells were analyzed by flow cytometry. Numbers in quadrants in (A) or beside windows in (B) indicate the percentage of the total lymphocyte population. A representative result out of three experiments is shown. In PBS-treated mice, the percentage of B220$^+$-cell population per total lymphocyte population was 69.3 ± 3.4% (*HS1*$^{+/+}$, n = 3) or 68.0 ± 6.3% (*HS1*$^{3'ko/3'ko}$, n = 3), and that in anti-IgM-treated mice was 16.1 ± 8.9% (*HS1*$^{+/+}$, n = 3) or 43.8 ± 3.3% (*HS1*$^{3'ko/3'ko}$, n = 3).

markedly to ~10% of those in *HS1*$^{+/+}$/H-Y Tg female mice (Table I). The population of DP cells in *HS1*$^{+/+}$/H-Y Tg male mice was almost entirely eliminated (Figure 8A). The residual DP cells mostly expressed non-transgenic TCR α-chain derived from endogenous α-chain gene allele; they thus lost the specificity to the H-Y antigen (Figure 8B) and a small population of thymocytes expressing CD8 at a low level (CD8^{low} SP cells) was observed (Figure 8A). In contrast, a significant population of the DP thymocytes remained undeleted in *HS1*$^{-/-}$/H-Y Tg male mice, together with an increased proportion of

Table I. Partial impairment of negative selection of thymocytes in HS1-deficient male mice

Mice	No. of thymocytes ($\times 10^7$)		
	Total	TgαTCR^{+a} CD8$^+$CD4$^+$	TgαTCR^{+a} CD8$^+$CD4$^-$
+/+ male (n = 9)	1.29 ± 0.41	0.08 ± 0.04	–
–/– male (n = 8)	2.16 ± 0.64	0.29 ± 0.13	–
+/+ female (n = 4)	10.1 ± 1.9	–	2.6 ± 0.3
–/– female (n = 4)	11.3 ± 2.2	–	2.9 ± 0.6

Thymocytes from *HS1*$^{3'ko/3'ko}$ mice (–/–) or their wild-type littermates (+/+), both being positive for anti-H-Y αβTCR transgenes, were analyzed as in Figure 8. Numbers (mean ± SD) of total thymocyes (left), transgenic αTCR$^+$ DP cells in male mice (middle) or transgenic αTCR$^+$ CD8$^+$ SP cells in female mice (right) are shown.
[a]Numbers were calculated as follows. Total nucleated cell numbers (Total) were multiplied by the percentage of either CD8$^+$CD4$^+$ fractions for male or CD8$^+$CD4$^-$ fractions for females (cells falling into the corresponding windows depicted in the contour profiles in Figure 8B), and the obtained values were multiplied by the percentage of Tg αTCR$^+$ fraction (cells falling into the regions defined by the bars drawn in the corresponding histograms in Figure 8B).

the CD8^{low} SP cells compared with those in *HS1*$^{+/+}$/H-Y Tg male thymocytes (Figure 8A). These DP and CD8^{low} SP cells in *HS1*$^{-/-}$/H-Y Tg male mice highly expressed the heat-stable antigen (HSA), indicating that they are in the immature stage of thymic development (data not shown). These cells expressed exclusively Tg β chains (data not shown) as a result of β-chain allelic exclusion (Uematsu *et al.*, 1988) and most of them also expressed Tg α chains (Figure 8B). Therefore, it is indicated that the DP cells lacking HS1 protein were able to escape the elimination process and accumulate in the thymus to a certain extent, despite the expression of anti-self Tg αβTCR on the cell surface. The phenotypic difference between the *HS1*$^{+/+}$ and *HS1*$^{-/-}$ Tg thymi was rather consistent. The number of DP thymocytes which expressed Tg TCR on the surface was ~3.6-fold more in *HS1*$^{-/-}$/H-Y Tg male mice than in *HS1*$^{+/+}$/H-Y male mice on average (Table I). This observation suggests that the HS1 protein is necessary for the complete elimination of the anti-self thymocytes induced by TCR cross-linking.

As reported in the case of H-Y Tg female mice (Kisielow *et al.*, 1988), the proportion of CD8$^+$ SP thymocytes expressing Tg α chain in *HS1*$^{+/+}$/H-Y Tg females increased compared with non-transgenic mice, as a result of positive selection (Figure 8A and B, and data not shown). The numbers of total thymocytes and CD8$^+$ SP thymocytes expressing Tg α chain from *HS1*$^{-/-}$/H-Y Tg females were equivalent to those from *HS1*$^{+/+}$/H-Y Tg females (Table I), indicating that the absence of HS1 protein had no apparent effect on the positive selection of thymocytes. It was noted that the expression level of CD8 was slightly lower on thymocytes from *HS1*$^{-/-}$/H-Y Tg females than those from *HS1*$^{+/+}$/H-Y Tg females [mean fluorescent intensity was 156.6 ± 3.0 (n = 4) in *HS1*$^{+/+}$/H-Y Tg females and 140.5 ± 7.8 (n = 4) in *HS1*$^{-/-}$/H-Y Tg females]. The significance of this matter is currently unclear.

Treatment of normal mice with glucocorticoids or γ-irradiation rapidly eliminates DP thymocytes. Such elimination was shown to be a result of apoptosis of the DP cells, and to be prevented by an overexpression of Bcl-2 protein which is known to protect a broad range of apoptosis (Sentman *et al.*, 1991; Strasser *et al.*, 1991b). To verify a possibility that the incomplete deletion of self-recognizing DP thymocytes in HS1-deficient mice stated above is due to a defect in a general machinery processing the apoptosis, we analyzed whether the DP thymocytes in *HS1*$^{-/-}$ mice are also resistant to such treatments. Forty-eight hours after i.p. injection of dexamethasone or whole-body γ-irradiation to either *HS1*$^{+/+}$ or *HS1*$^{-/-}$ mice, thymi were removed and thymocyte subsets were analyzed by flow cytometry. As shown in Figure 9, DP thymocytes were eliminated in *HS1*$^{-/-}$ mice to the same extent as those in *HS1*$^{+/+}$ mice by γ-irradiation, or even more by dexamethasone. This suggests that HS1 is not involved in a general process of apoptosis of thymocytes, but rather in a specific pathway leading to the apoptosis that is initiated at cross-linked antigen receptors.

Discussion

HS1 is involved in an antigen-receptor-derived signaling pathway as a substrate of NR-PTK(s)

HS1 was shown to be one of the major substrates of NR-PTK(s) activated by cross-linking of sIgM in a B-lymphoma line. We described here that HS1 was rapidly tyrosine phosphorylated after cross-linking of sIgM on normal splenic B cells, or TCR–CD3 complex on splenic T cells. It was also shown that HS1 had strong affinity to SH2 domains of *src* family tyrosine kinases such as Lyn, Fyn and Blk only after cross-linking of sIgM in another B-lymphoma line (Baumann *et al.*, 1994). Taken together with the present results that the responses of B and T cells induced by such antigen-receptor cross-linking were partially impaired in HS1-deficient mice, it is indicated that HS1 is involved functionally in the signaling pathways from the antigen receptors as a substrate of NR-PTK(s).

In lymphocytes, the antigen-receptor cross-linking appears to be a rather specific stimulus that induces the tyrosine phosphorylation of HS1. Other stimuli, such as LPS, soluble CD40L or IL-4, did not induce the phosphorylation of HS1 in B cells. Accordingly, proliferative responses of B cells to these stimuli were not affected by the absence of HS1 protein, nor were those of T cells to concanavalin A (ConA), phytohemagglutinin (PHA) or IL-2 (data not shown). Therefore, HS1 appears to be specifically associated with antigen-receptor-induced signals in lymphocytes. However, it has recently been reported that tyrosine phosphorylation of HS1 is induced by IL-5 in an IL-5-dependent mouse precursor B-cell line (Sato *et al.*, 1994), or by cross-linking of FcεRI in a mouse mast cell line and bone marrow-derived mast cells (Fukamachi *et al.*, 1994), indicating that HS1 may be involved in signal transduction from those receptors in such types of cells.

In the HS1-deficient mice, the proliferative response to antigen-receptor cross-linking was impaired in both splenic B and T cells, suggesting that the similar signaling pathway leading to the proliferation, in which HS1 is involved, exists in both B and T cells. The defect in the B-cell proliferative response to sIgM cross-linking was also shown in *xid* mice (Scher, 1982), which have a mutation in the gene encoding for Btk (Rawlings *et al.*, 1993; Thomas *et al.*, 1993), whereas the defect in the T-cell

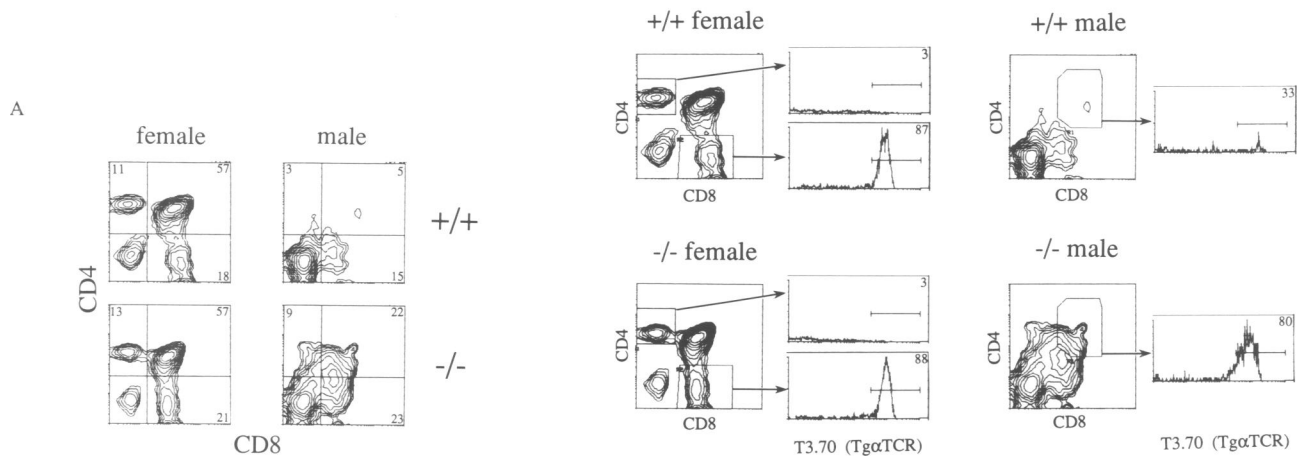

Fig. 8. Impaired negative selection of anti-H-Y αβTCR transgenic thymocytes in HS1-deficient male mice. HS1-deficient mice (*HS1*$^{3'ko/3'ko}$) were crossed with mice bearing a transgene coding for a TCR against the H-Y (male) antigen as described in Materials and methods. All mice used for the experiment shown here were transgene positive. Thymocytes from 4- to 8-week-old *HS1*$^{3'ko/3'ko}$ (–/–) mice or their wild-type littermates (+/+) carrying the transgenes were stained with PE–anti-CD4, FITC–anti-CD8 and biotinylated transgenic TCR α-chain (Tg αTCR)-specific MoAb (T3.70) plus streptavidin–RED670. The stained cells were analyzed by flow cytometry. Expression of CD4 versus CD8 antigens is shown as a contour profile in each panel (**A**). Numbers in quadrants indicate the percentage of the total lymphocyte population. Expression of Tg αTCR on the surface of $CD8^+$SP or $CD4^+$SP thymocytes in females, and of DP thymocytes in males (cells falling into the windows drawn in each panel), is shown as a histogram on the right of each panel (**B**). The percentage of Tg αTCR-positive cells (bars) with respect to the total cells falling into each window is denoted in the histograms.

proliferation by TCR cross-linking was shown in the mice lacking Lck or Fyn proteins in lymphocytes (Appleby *et al.*, 1992; Molina *et al.*, 1992; Stein *et al.*, 1992). Also, in the mice lacking exon 6 of the gene coding for CD45, which presumably upregulates the activity of NR-PTKs in lymphocytes, both B and T cells exhibited the impaired responses to the cross-linking of antigen receptors, suggesting that the activation of the NR-PTKs is necessary for the responses of these cells (Kishihara *et al.*, 1993). Taking these together, one could presume that the impaired proliferative responses to the antigen-receptor cross-linking of B or T cells in the mice with deficiency in NR-PTKs may be due, at least in part, to the lack of induction of tyrosine phosphorylation and activation of HS1 protein. It is in our interest to check the tyrosine phosphorylation of HS1 protein in the lymphocytes of these mutant mice.

The proliferative responses and the immune response to T-independent antigens were not completely impaired in the HS1-deficient mice. This may indicate the presence of redundant pathways of signaling from antigen receptors leading to these responses of the cells. Similarly, functional redundancy of NR-PTKs was reported to mask the expected phenotypes in *fyn*, *src* or *yes* gene knock-out mice (Appleby *et al.*, 1992; Stein *et al.*, 1994). Ras-mediated pathway could be one of the alternatives to the HS1-mediated pathway, since it was shown that guanine-nucleotide releasing factors, such as Sos and Vav, were potentiated after the antigen-receptor cross-linking and could activate Ras by converting it into a GTP-associated form (Gulbins *et al.*, 1993; Harwood and Cambier, 1993; Lazarus *et al.*, 1993; Saxton *et al.*, 1994). It is also possible that substrates of NR-PTKs other than Vav or Shc, to which Sos is physically linked through an adaptor molecule, Grb2, may compensate for a part of the function of HS1 in the signaling pathway. Alternatively, HS1 might function to enhance the signals mediated through the other molecules, such as Ras.

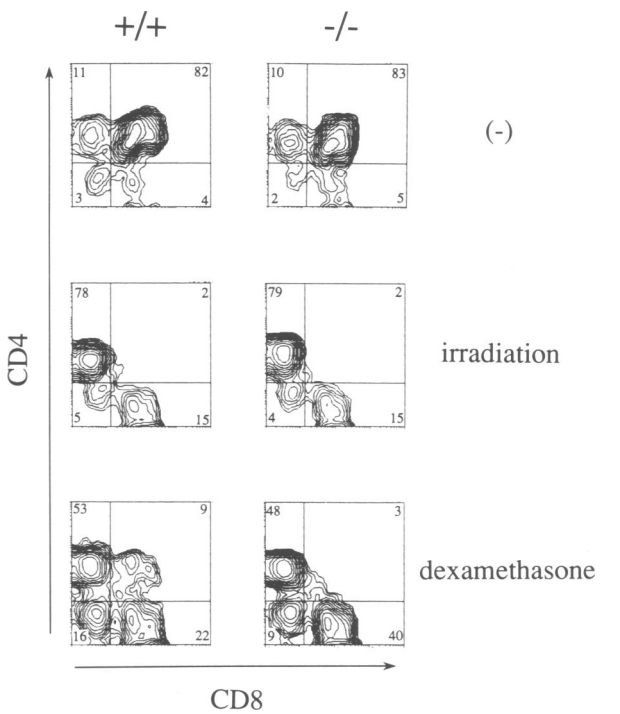

Fig. 9. Sensitivity of thymocytes in HS1-deficient mice to administration of dexamethasone or γ-irradiation. *HS1*$^{3'ko/3'ko}$ mice (–/–) or their wild-type littermates (+/+) (8 weeks old) were non-treated (–), injected i.p. with dexamethasone (0.5 mg per mouse) or γ-irradiated (9 Gy). After 48 h, the animals were sacrificed, and thymocytes were stained with PE–anti-CD4 and FITC–anti-CD8, then analyzed by flow cytometry. Expression of CD4 versus CD8 antigens is shown as a contour profile in each panel. Numbers in quadrants indicate the percentage of the total lymphocyte population.

Role of HS1 in antigen-receptor-induced apoptosis of B cells

Recent studies showed that peritoneal B cells were eliminated by apoptosis *in vivo* upon cross-linking of sIgM with its specific antigen (Murakami *et al.*, 1992) or with antibody against sIgM (Tsubata *et al.*, 1994). Here, we demonstrated that the peritoneal B cells in HS1-deficient mice were resistant to the treatment with anti-IgM antibody, indicating that the lack of HS1 impairs the signaling from sIgM leading to the apoptosis. This result is consistent with our recent finding from the following experiment. From murine B-lymphoma WEHI-231, which is known to undergo apoptosis upon cross-linking of sIgM, the variants resistant to the sIgM cross-linking were isolated (Hibner *et al.*, 1993). These cells were shown to express HS1 protein at a markedly reduced level (Benhamou *et al.*, 1994), and introduction of the vector expressing HS1 protein into one of these variants restored its sensitivity to sIgM cross-linking (Fukuda *et al.*, 1995), indicating that the HS1 is necessary for the antigen-receptor-induced apoptosis in this immature B-cell line.

The deregulated expression of an exogenously introduced *bcl-2* gene was shown to prevent apoptosis induced by sIgM cross-linking of peritoneal B cells in both normal mice (Tsubata *et al.*, 1994) and mice carrying the immunoglobulin transgenes for the anti-erythrocyte antibody (Nisitani *et al.*, 1993). Therefore, it is possible to speculate that HS1 may act as a suppressor of Bcl-2 so that the resistance of the HS1-deficient cells might be caused by upregulation or activation of Bcl-2. However, it was shown that the apoptosis of WEHI-231 cells induced by sIgM cross-linking, but not by heat shock, was uninhibitable by overexpression of the introduced *bcl-2* gene (Cuende *et al.*, 1993). In addition, the variants of WEHI-231 cells, which expressed very little HS1 and were resistant to the cross-linking of sIgM, expressed Bcl-2 at the same level as the parental WEHI-231 cells (Hibner *et al.*, 1993). These results make the above possibility unlikely. Rather, the HS1 protein may be involved directly in an apoptotic signaling pathway which is not regulated by Bcl-2. This hypothesis is supported by the facts that spontaneous cell death of splenic B cells *in vitro* (data not shown), or apoptosis of thymocytes induced *in vivo* by irradiation or steroid injection, still occurred in the HS1-deficient mice at equivalent levels to the control mice (Figure 9), although overexpression of Bcl-2 prevented the apoptosis in such cases (Sentman *et al.*, 1991; Strasser *et al.*, 1991a,b).

Negative selection of thymocytes in HS1-deficient mice

In contrast to the thymus of $HS1^{+/+}$/H-Y Tg male mice, where DP or $CD8^+$SP thymocytes expressing the Tg αβTCR were virtually absent (Kisielow *et al.*, 1988), that of $HS1^{-/-}$/H-Y Tg male mice retained a significant number of DP cells expressing Tg αβTCR on their surface (Figure 8 and Table I), indicating that they had escaped from the elimination. The population of $CD8^{low}$ SP thymocytes was also present more in $HS1^{-/-}$/H-Y male mice than in $HS1^{+/+}$/H-Y male mice. High expression of HSA on these cells indicates that they are the cells in the developmental stage between DN thymocytes and early DP thymocytes (Paterson and Williams, 1987; MacDonald *et al.*, 1988). Thus, it is indicated that HS1 is necessary for the elimination of self-reactive thymocytes in the stage where DN thymocytes differentiate into early DP thymocytes.

On the other hand, the present data also showed that the elimination of the Tg TCR^+ thymocytes in the later developmental stages, namely DP cells expressing CD4 and CD8 at high level (late DP cells) and mature SP cells, did occur in spite of the absence of HS1 protein. This differential requirement for HS1 on the elimination of self-reactive thymocytes may be explained by the differential expression of CD8 on the cell surface. In the H-Y Tg system, it was shown that self-reactive thymocytes were partially rescued from negative selection when the mutation of $H\text{-}2D^b$ molecules inhibited their association with CD8 (Killeen *et al.*, 1992), or when CD8 molecules were absent on the surface (Fung-Leung *et al.*, 1993). Thus, the association of the CD8 molecules with the MHC class I molecules appears to be necessary for the efficient negative selection of thymocytes in this system. The expression level of CD8 is thought to correlate to the avidity of the TCR complex to peptide/MHC complex, which is critical to determine the T-cell fate (Robey *et al.*, 1991, 1992). At the affinity of the Tg TCR for the H-Y peptide/MHC complex, self-reactive thymocytes with low-level expression of CD8 were eliminated in $HS1^{+/+}$ male mice and could not differentiate into the early DP stage. However, such cells were not eliminated in $HS1^{-/-}$ males and could differentiate into early DP stage, possibly because the signal through the TCR–CD3 complex was insufficient to initiate cell death. On the other hand, the self-reactive late DP thymocytes were eliminated in the $HS1^{-/-}$ mice, possibly because high-level expression of CD8 increased the avidity of the TCR–CD3 complex to the peptide/MHC complex, generating sufficient signals for the induction of cell death despite the absence of HS1. In this regard, HS1 may play a role in enhancing the signals responsible for cell death at the early stage of thymocyte development.

Another explanation for the partial impairment of thymic negative selection in HS1-deficient mice might be the existence of different mechanisms for the selection at different developmental stages. It was previously shown that the DN thymocytes expressing anti-H-Y αβTCR were not deleted even when transgenic CD8α chain was expressed at high level (van Oers *et al.*, 1993). This suggests that the susceptibility of thymocytes to negative selection may not only be affected by the avidity of the TCR–CD3 complex and the peptide/MHC complex, but may also be determined by their developmental stages. Thus, dependency of the negative selection on HS1 may be controlled by the developmental stages of thymocytes regardless of their CD8 expression.

Possible mechanisms of how HS1 works

Here we demonstrated that HS1, a substrate of NR-PTK(s), is one of the functional components in signaling pathways from antigen receptors that lead to the responses such as proliferation or clonal deletion of B and T cells. Supposing that these responses require new protein synthesis (Ishida *et al.*, 1992), one may speculate that HS1 regulates the transcription of some gene(s) which regulates the fate of lymphocytes.

Experiments using antisense oligonucleotides suggested

that apoptosis induced by cross-linking of sIgM on B lymphomas requires an induction of Blk kinase (Yao and Scott, 1993) and a rapid depression of induced expression of a nuclear transcriptional factor, c-myc (Fischer *et al.*, 1994). Thus, it may be possible to assume that there are molecules which are phosphorylated by the NR-PTK(s), like Blk, upon sIgM cross-linking and transmit the signal to the nucleus, then regulate the transcription of the target genes, e.g. c-*myc*, which are responsible for the following events that eventually lead to the cellular responses.

Recently, a new family of transcription factors, the STAT family, has emerged. After exposure to various external stimuli such as interferons, interleukins or growth factors, the STAT proteins are phosphorylated by Jak-Tyk family tyrosine kinases in the cytoplasm, and translocate into the nucleus to regulate the transcription of their target genes (Fu, 1992; Shuai *et al.*, 1992, 1993; Bonni *et al.*, 1993; Kotanides and Reich, 1993; Larner *et al.*, 1993; Lutticken *et al.*, 1993; Ruff-Jamison *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993). Although its DNA-binding or *trans*-acting activity has not yet been proved, the HS1 protein possesses several sequence motifs characteristic of transcription factors (Kitamura *et al.*, 1989), namely repeating motifs containing a helix–turn–helix structure similar to those in several prokaryotic transcription factors (Laughon and Scott, 1984), a sequence similar to a consensus for DNA-binding motifs found in the proteins such as Jun-, Fos-, Myc- and other helix–loop–helix protein families (Prendergast and Ziff, 1989), and the acidic and amphipathic α-helix (Ptashne, 1988). The HS1 protein localizes in the cytoplasm and, to a lesser extent, in the nucleus, and the amount of tyrosine-phosphorylated HS1 protein in the nucleus markedly increases after cross-linking of sIgM (unpublished data). These observations support the idea that HS1 protein might act as a shuttle molecule between the cytoplasm and the nucleus, and transmit the signal from the antigen receptor-associated NR-PTK(s) to the target gene(s), in a fashion similar to the STAT proteins.

Materials and methods

Targeting vectors

The pHS1 5′ko vector was constructed as follows. From the plasmid pEE7.5, carrying a 7.5 kb *Eco*RI–*Eco*RI *HS1* gene fragment isolated from a Balb/c genomic library, a 1.4 kb *Xba*I–*Bgl*II fragment containing exon II of *HS1* gene was recovered and inserted into the *Xba*I–*Bam*HI site of pBluescriptIISK(+) (pBS, Stratagene) to make plasmid pXB1.4. In the pXB1.4 vector, a region from a *Hin*cII site located just upstream of exon II down to a *Xho*I site in the multi-cloning site (MCS) of the pBS vector was removed and exchanged with a neomycin-resistant (*neo*^{r}) gene released from pMC1NeoPolyA (Stratagene) with *Hin*cII and *Xho*I. From the resultant vector, a 2.1 kb *Xba*I–*Xho*I fragment containing a 5′-end homologous region and the *neo*^{r} gene was isolated, and inserted by blunt-end ligation into a blunt-ended *Cla*I site of the pHE4 vector, which carried in the pBS a 4.0 kb *Hin*dIII–*Eco*RI fragment taken from pEE7.5 corresponding to 3′-end homologous region, to produce the pXNeoE. The MC1-TK cassette (Mansour *et al.*, 1988), released from pMC1TK (gift of Dr Katsuki) by *Xho*I–*Bam*HI digestion, was blunt-ended and ligated into the *Hin*cII site of pUC18 to make pUCTK. Finally, a *Bam*HI–*Hin*dIII MC1-TK fragment from the pUCTK was blunt-ended and inserted into a *Sma*I site of pXNeoE.

The pHS1 3′ko vector was constructed as follows. The starting vector was pEE6.5, which carried a 6.5 kb *Eco*RI–*Eco*RI *HS1* gene fragment isolated from a Balb/c genomic library. Using the pEE6.5 as a template, a 0.9 kb genomic fragment was amplified by PCR with a 5′-end primer (5′-CTGAGAGGAAAGGTAGACACCAGG-3′) and a 3′-end primer (5′-CTTGCCATGGGCACCACTGGAAGCT-3′) containing a *Nco*I site for the ligation to a lac-Z cassette (see below), at an annealing temperature of 60°C. The product was digested with *Hin*dIII, which locates at 45 bp downstream of the 5′-end of the amplified region, and *Nco*I, then trimolecular ligated with the *Nco*I–*Bam*HI fragment of a lac-Z cassette released from pactbgal (gift of Dr Yagi) and a *Hin*dIII–*Bam*HI fragment of pBS. As confirmed by DNA sequencing, ligation at the *Nco*I site generated three stop codons to terminate HS1 translation and placed a start codon for the *lac-Z* gene at 5′ to these stop codons in a frame shifted from that of the HS1 translation. From the resultant vector, a 4.7 kb fragment containing the amplified genomic region and lac-Z cassette was released by *Xho*I and *Xba*I digestion, and blunt-ended with Klenow enzyme. A 5 kb *Bgl*II–*Hin*dIII *HS1* gene fragment from pEE6.5, which corresponds to the 3′-end homologous region, was inserted into the *Bam*HI–*Hin*dIII site of pMC1Neo (Stratagene) to make pNeoBH5. Finally, the 4.7 kb blunt-ended fragment mentioned above was inserted into the *Sma*I site of the pNeoBH5.

The HS1 5′ko vector was linearized with *Xho*I and the pHS1 3′ko vector with *Hin*dIII, before transfection into ES cells.

Generation of HS1 gene targeted mice

ES cell line E14 (Hooper *et al.*, 1987) was cultured, transfected and screened as previously described (Kitamura *et al.*, 1991). After transfection of the pHS1 5′ko vector, 518 colonies resistant to both G418 and GANC were screened for the homologous recombination event by PCR with a primer located upstream of the short arm of the homology (5′-TCTTCCTCAGATGTCTTCGG-3′), and a primer complementary to the *neo*^{r} gene sequence (5′-TGGGTCGTTTGTTCGG-ATCC-3′), at an annealing temperature of 63°C. As for the pHS1 3′ko vector, 252 G418-resistant colonies were screened by PCR with a primer located upstream of the short arm of the homology (5′-CTGAGAGG-AAAGGTAGACACCAGG-3′) and a primer complementary to the *lac-Z* gene sequence (5′-CATGCTTGGAACAACGAGCGCAGC-3′), at an annealing temperature of 63°C. The precise homologous recombination was confirmed by Southern blot analysis for each case. In the case of pHS1 5′ko, genomic DNA from PCR positive clones was digested with *Eco*RI, and hybridized with a 0.9 kb *Eco*RI–*Bam*HI fragment (probe A shown in Figure 1) or a 1.0 kb *Bgl*II–*Bgl*II fragment (probe B). In the case of pHS1 3′ko, genomic DNA was digested with *Xba*I and hybridized with a 1.5 kb *Eco*RI–*Eco*RI fragment (probe C) or digested with *Eco*RI and hybridized with a 0.8 kb *Hin*dIII–*Bam*HI fragment (probe D). Seven colonies containing the desired $HS1^{5'ko}$ allele and 28 containing the desired $HS1^{3'ko}$ allele were identified. Hybridization with the *neo*^{r} probe verified the presence of a single copy of the *neo*^{r} gene for each clone.

ES cells carrying the targeted *HS1* allele were injected into blastocysts of C57BL/6 mice as described (Bradley, 1987); the resulting male chimeras were mated to C57BL/6 females, and their agouti offspring were screened by PCR and Southern blot analysis to detect the heterozygous mutation in the genome using DNA from their tails. One out of four injected ES clones was able to transmit the $HS1^{5'ko}$ allele into the germline, and all four to transmit the $HS1^{3'ko}$ allele into the germline. The heterozygotes were intercrossed to produce homozygous offspring.

Antibodies

A *Pvu*II–*Ban*I (converted to blunt end) fragment corresponding to the repeating motifs (Leu^{57}–Ser^{233}) of the mouse HS1 cDNA was cloned into the *Sma*I site of pGEX-3X (Pharmacia). The glutathione-S-transferase (GST) fusion protein was produced from this vector in bacteria and purified on a glutathione–Sepharose 4B column as recommended by the supplier (Pharmacia). An adult New Zealand white rabbit was immunized with 1 mg of the fusion protein emulsified with complete Freund's adjuvant, followed by boosting twice with incomplete Freund's adjuvant. The immune serum (anti-repeat) was depleted of antibody against GST protein by passing through the GST-protein-bound glutathione–Sepharose column, and purified by an affinity column containing the GST–HS1 fusion protein coupled to Tresyl-activated Sepharose 4B (Pharmacia). Rat antiserum (anti-peptide) was against a synthetic peptide corresponding to amino acids 306–320 of mouse HS1.

MoAbs against CD3ε (145.2C11; Leo *et al.*, 1987), the transgenic TCR α chain (T3.70; Teh *et al.*, 1988) and HSA (M1/69; Springer *et al.*, 1978) were purified from hybridoma culture supernatants using a protein-G column. T3.70 was biotinylated and M1/69 was conjugated with fluorescein isothiocyanate (FITC). Anti-mouse IgM MoAb (AK9) and anti-rat κ MoAb (MAR18.5) were kind gifts from Dr T.Tsubata, and anti-mouse IgM MoAb (B.7.6) was a kind gift from Dr C.Paige. Other MoAbs used in this paper are: horseradish-peroxidase (HRP)-labeled anti-phosphotyrosine MoAb, PY20 (Transduction), FITC– or biotinylated

anti-Lyt2 (CD8), phycoerythin (PE)-anti-L3T4 (CD4) and FITC–anti-Thy1 (Becton Dickinson), FITC– or PE–anti-B220 (Coulter) and PE–anti-Ly1 (Pharmingen). Streptavidin–RED670 (Gibco BRL) was used as a secondary reagent for the biotinylated antibodies. Polyclonal antibodies are: affinity-purified goat anti-mouse IgM, goat anti-mouse IgG Fc and FITC–anti-mouse IgM (Cappel), affinity-purified goat anti-mouse IgM $F(ab')_2$ fragment (Southern Bio Technology), HRP-labeled goat anti-rabbit IgG and HRP-labeled rabbit anti-rat IgG (Zymed).

Flow cytometric analysis

Splenocytes, thymocytes, bone marrow cells and peritoneal exudate cells were stained with antibodies mentioned above as described previously (Kishi *et al.*, 1991), and analyzed with FACScan cytometer using the Lysis program (Becton-Dickinson) or EPICS XL cytometer (Coulter). Living cells present in the lymphocyte gate defined by light scatter (Forster *et al.*, 1989) were analyzed.

Purification of cells

Splenic B cells were purified from spleens using a previously described method that entailed killing T cells with culture supernatant of hybridoma T24 (anti-Thy1 MoAb; Dennert *et al.*, 1980) plus rabbit complement (Cedarlane), and small, dense B cells were further purified by fractionation on discontinuous gradients of Percoll (Pharmacia) as described previously (Nathanson *et al.*, 1977). The resulting preparations were typically 90% B220 positive. Splenic T cells were purified by enrichment with a nylon wool column, followed by depletion of B and adherent cells by incubation at 37°C for 1 h on Petri dishes coated with affinity-purified goat anti-mouse IgM polyclonal antibody. The remaining cells were typically 80% Thy-1 positive. Peritoneal B cells were enriched by depletion of adherent cells by incubation on Petri dishes at 37°C for 1 h.

Cell culture

Small, dense B cells or splenic T cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (Gibco Laboratories), L-gulutamine (2 mM) and 2-mercaptoethanol (50 mM). For proliferation assays, B cells (1×10^5/well) were cultured in 96-well flat-bottom tissue culture plate (Falcon) either alone or in the presence of *Salmonella typhimurium* LPS (1 μg/ml; Difco Laboratories), culture supernatant (33% volume) of cells producing murine CD40L–CD8 chimeric molecule (Lane *et al.*, 1993), affinity-purified goat anti-mouse IgM (5 μg/ml; Cappel), affinity-purified goat anti-mouse IgM $F(ab')_2$ fragment (5 μg/ml; Southern Bio Technology) or anti-mouse IgM MoAb, B-7-6 (1, 2, 4 or 8 μg/ml). Splenic T cells (1×10^5/well) were cultured in 96-well culture plate in the absence or presence of plastic-immobilized anti-CD3ε MoAb, 145.2C11 (0.25, 1 or 4 μg/ml). Cultured cells were pulsed for the last 6 h of a 48 or 72 h culture period with 1 μCi/well of [^{3}H]thymidine (Dupont MEN). Cells were then harvested onto glass fiber filters and radioactivity incorporated into DNA was determined by standard method.

Immunoblot analysis

To detect the HS1 protein, splenocytes (10^8 cells/ml) were lyzed in 1× SDS–PAGE buffer [62 mM Tris–HCl (pH 7.5), 2% SDS, 1 mM EDTA, 10% glycerol, 5% 2-mercaptoethanol] and sonicated with SONIFIER 250 (Branson). The lysates were cleared by centrifugation, and 30 μl of each of them were loaded onto a 10 or 8% SDS–polyacrylamide gel, then electrotransferred to a nitrocellulose filter by the semi-dry method. After blocking with 5% non-fat milk in TBST [20 mM Tris, 150 mM NaCl, 0.05% Tween 20 (pH 8.0)], the filter was incubated with antisera against mouse HS1 (rabbit anti-repeat or rat anti-peptide) in the blocking buffer followed by incubation with HRP-labeled goat anti-rabbit IgG or rabbit anti-rat IgG, and developed with the ECL system (Amersham).

To detect tyrosine-phosphorylated proteins, 5×10^6 splenic B cells or 1×10^6 peritoneal B cells were resuspended in 0.2 ml of RPMI-1640 medium. After preincubation at 37°C for 5 min, affinity-purified goat anti-mouse IgM polyclonal antibody (25 μg/ml) was added. Splenic T cells (5×10^6 cells in 50 μl of RPMI-1640 medium) were incubated with 20 μg/ml of the anti-CD3ε MoAb (145.2C11) on ice for 10 min, then the second antibody, goat anti-mouse IgG (20 μg/ml), was added and cells were transferred into 37°C to start the reaction. The cells were incubated for various lengths of time and stopped by adding ice-cold phosphate-buffered saline (PBS) containing 2 mM sodium orthovanadate. After washing once with the PBS–orthovanadate, cell pellets were lyzed in 50 μl 1× SDS–PAGE buffer containing 2 mM sodium orthovanadate. The cell lysates were cleared by centrifugation, electrophoresed and transfered to a nitrocellulose filter as above. After blocking with 3% BSA–TBST containing 2 mM sodium orthovanadate, the filter was incubated with the HRP-labeled PY20 in the blocking buffer, and developed with the ECL system.

The filters were stripped of bound antibodies by incubation at 50°C for 30 min in a buffer [100 mM 2-mercaptoethnol, 2% SDS, 62.5 mM Tris–HCl (pH 6.7)], and reprobed with the anti-repeat antibody.

Immune response to T-dependent and T-independent antigens

TNP_{51}-Ficoll, the type II T-independent antigen, was kindly provided by Dr Ono. A total of 20 μg of TNP_{51}-Ficoll in saline was injected i.p. into each mouse. The sera were collected at the indicated time points shown in Figure 6, and the titer of TNP-binding antibody was determined by enzyme linked immunosorbent assay (ELISA). Briefly, plastic plates were coated with TNP–BSA (10 μg/ml) in PBS at 4°C overnight, followed by blocking with 5% non-fat milk in TBST. After washing the plates with TBST, diluted serum samples were added to the plates. The bound IgM was detected with HRP-labeled goat anti-mouse IgM antibody (TAGO) and developed with TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories).

A total of 75 μg of NP_{14}-chicken gamma globulin (a kind gift of Dr Azuma) in complete Freund's adjuvant was injected i.p. into each mouse. The mice were boosted 12 days later with 75 μg of the same antigen in incomplete Freund's adjuvant. Sera were collected at the indicated time points shown in Figure 6 and the titer of chicken gammaglobulin (CG)-binding antibody was determined by ELISA as indicated above with CG (10 μg/ml)-coated plastic plates, except that the bound IgG was detected with HRP-labeled goat anti-mouse IgG antibody (Zymed).

Intraperitoneal antibody injection

A total of 150 μg of the anti-mouse IgM MoAb (AK9, rat IgG) and, after 30 min, 150 μg of the anti-rat κ MoAb (MAR18.5, a second antibody), were injected into the peritoneal cavity of 4- to 6-week-old mice as described previously (Tsubata *et al.*, 1994). Twenty-four hours later, peritoneal exudate cells were recovered by washing the peritoneal cavity with PBS, stained as above and analyzed by flow cytometry.

Mice

Anti-H-Y αβTCR transgenic mice on the C57BL/6 background (H-2^b; Kisielow *et al.*, 1988), kindly provided by Dr H.S.Teh, were bred with $HS1^{3'ko/3'ko}$ mice [(129/Ola×C57BL/6) F2, H-2^b]. The offspring were intercrossed to obtain $HS1^{+/+}$, $HS1^{3'ko/+}$, $HS1^{3'ko/3'ko}$ mice containing the transgene. The presence of the transgene in the genome was determined with DNA from tails by PCR using primers (5′-ACAA-GGTGGCAGTAACAGGA-3′ and 5′-ACAGTCAGTCTGGTTCCTGA-3′) at an annealing temperature of 60°C. The *HS1* alleles were determined by Southern blot analysis as described before with the same DNA samples.

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